Bacillus subtilis at near-zero specific growth rates

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Summary and Discussion

Overview

In conditions with low nutrient availability, microorganisms are considered to grow at rates that approach zero. In this thesis, the physiological- and transcriptional response of *Bacillus subtilis* to near-zero growth rates during retentostat cultivation was studied.

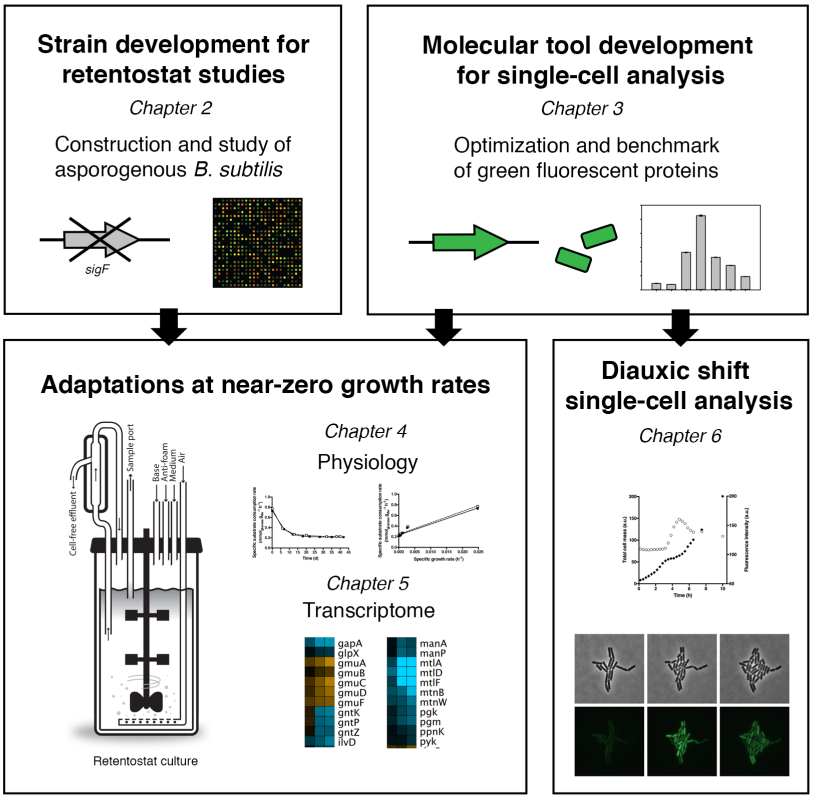

Figure 1. Thesis overview.

The starvation-induced non-growing conditions during stationary phase in batch culture have been subject of numerous studies (Banse et al., 2008; Bernhardt et al., 2003; Blom et al., 2011; Britton et al., 2002; Koburger et al., 2005; Navarro Llorens et al., 2010; Nyström, 2004; Robleto et al., 2007). However, the zero-growth condition, in which there is still energy available for maintenance processes to be

carried out, is transient in this culturing method and therefore difficult to study. Nevertheless, by using retentostat cultivation, near-zero growth rates have become experimentally accessible and have been subject of investigation in various microorganisms (Arbige and Chesbro, 1982; Bisschops et al., 2014; Boender et al., 2009; Ercan et al., 2013; Goffin et al., 2010; Tappe et al., 1996, 1999; van Verseveld et al., 1984, 1986). In this thesis the first retentostat cultivation with the soil-dwelling bacteria *B. subtilis* is described. Therefore, this thesis not only focuses on the cellular response to extremely low growth rates, but also on the experimental setup for aerobic retentostat cultivation. An overview of the research described in this chapter is depicted in Fig. 1.

Maintenance energy requirement at near-zero growth rates

Already in 1898 it was postulated by Ducleaux, and later reestablished by Marr et al. (1963) and Pirt (1965), that part of the energy-substrate consumed by cells is used for maintenance purposes in a growth-rate independent way (Duclaux, 1898). The remaining part is used for biomass synthesis, which consequently is growth-rate dependent. Therefore, maintenance requirements become relatively more important at low specific growth rates. Schultz and Gerhardt (1969) described that in cultures with biomass retention, the ultimate biomass attainable will be determined by the maintenance energy requirement of the cells. Retentostat cultivation, first presented by Herbert (1961) and later refined by Pirt and Kurowski (1970), theoretically allows sustained cultivation in this condition where cells only expend energy for maintenance purposes, leading to specific growth rates of (near-)zero. This is achieved by means of a constant amount of supplied energy-substrate in combination with the retention of proliferating biomass. In 1979, the first retentostat study with the goal to bring a microbial culture to the growth limit imposed by the maintenance energy requirement of its cells, was published (Chesbro et al., 1979). The conclusion that available energy is always divided between maintenance and growth, but that maintenance processes cannot command the total supply, is a deviation from the concept of growth-rate-independent maintenance energy requirement. In the years after, various retentostat studies on other microorganisms followed (Arbige and Chesbro, 1982; Tappe et al., 1996, 1999; van Verseveld et al., 1984, 1986) and the concept maintenance energy requirements remained under debate (van Bodegom,

2007). At low- to intermediate specific growth rates, the growth-rate independent maintenance energy requirements of microorganisms adequately describe the stoichiometry of biomass and product formation (Tijhuis et al., 1993). However, it remained unclear to what extent this concept is applicable at (near-)zero growth rates. Nevertheless, the retentostat provided a means to study microbial behavior at very low growth rates.

With the advent of genomics techniques a renewed interest in near-zero growth conditions emerged. This led to a multi-laboratory collaboration within the Kluyver Centre for Genomics of Industrial Fermentation, with the goal of analyzing 'zero-growth' of industrial microbes. In 2009, the first energy-limited retentostat study from this initiative was published, analyzing the physiological response of *Saccharomyces cerevisiae* at near-zero growth rates (Boender et al., 2009). This and other retentostat studies on *Lactobacillus plantarum* (Goffin et al., 2010), *Lactococcus lactis* (Ercan et al., 2013), revealed that m_s values calculated from near-zero growth retentostats were in coherence with those extrapolated from higher growth rate chemostats. The conclusion that the maintenance energy requirement of these organisms is independent of the growth rate (Boender et al., 2009; Ercan et al., submitted, 2013; Goffin et al., 2010), is in contrast to the observation that maintenance requirements are reduced at low specific growth rates as is seen in the earlier retentostat studies (Arbige and Chesbro, 1982; Tappe et al., 1996, 1999; van Verseveld et al., 1984, 1986). Most likely the different outcomes of the newer studies are accountable to the fact that long-term retentostat cultivation (22 – 45 days) is applied, in contrast to the earlier studies, which applied short-term setups (3 days), not truly allowing zero-growth conditions to establish.

Adaptation of *B. subtilis* to near-zero growth rates

The increase of the carbon- and energy-source fraction used for maintenance processes by *B. subtilis* retentostat cultures from an initial 31% to 98%, corroborates with the progression from a growing state to a near-zero growth state. The virtually identical maintenance coefficient calculated from retentostat cultivation and from extrapolated chemostat cultures at higher growth rates, indicates a growth-rate independency of the maintenance energy requirements at (near-)zero growth rates (**Chapter 4**). This in line with other organisms studied in retentostats (Ercan et al.,

submitted) and suggests that *B. subtilis* does not adjust its maintenance energy requirements to sustain growth under the extreme caloric restriction in the retentostat. However, *B. subtilis* did express a number of carbon import and utilization systems, suggesting an adjustment of its active metabolic repertoire (**Chapter 5**). This gradual alleviation of carbon catabolite repression (CCR) when approaching zero-growth is a common response among various microorganisms (Boender et al., 2011; Ercan et al., submitted, 2014; Goffin et al., 2010) and also observed during diauxic growth and stationary phase (Blencke et al., 2003; Fujita, 2009; Kotte et al., 2014; Solopova et al., 2014). The anabolic demand is greatly reduced under (near-)zero growth conditions and this is reflected in the transcriptome by a decreased expression of genes involved in biosynthetic routes. This reduction of the translation machinery found in **Chapter 5**, is also seen in *S. cerevisiae* and *A. niger* (Boender et al., 2011; Jørgensen et al., 2010). The observed adaptations, which support survival rather than growth, are coherent with an increased focus of overall cellular activity to maintenance-associated processes. That it is an efficient response to the severe caloric restriction encountered in the retentostat is marked by the high culture viability (99%) throughout the cultivation. Such high viability is also observed in other retentostat studies (Boender et al., 2009; Ercan et al., 2013; Goffin et al., 2010; Jørgensen et al., 2010).

Although expression related to the hallmark response for stress resistance in *B. subtilis*, sporulation, is not detected in the transcriptome, carbon-limited chemostat cultivation is reported to already induce sporulation (Dawes and Mandelstam, 1970). Most likely a fraction of the culture has initiated sporulation in a retentostat, as it is a risk-spreading strategy (Fujita and Losick, 2005; de Jong et al., 2010; Veening et al., 2008a). *B. subtilis* is known for exhibiting various phenotypes within an isogenic population, especially during stationary phase (Branda et al., 2001; Dubnau, 1991; Errington, 2003; Kearns and Losick, 2005; Msadek, 1999; Smits et al., 2005; Veening et al., 2008a, 2008b, 2008c), and many processes under (near-)zero growth conditions could potentially exhibit this heterogeneity. However, whether regulation on the areas described above is prone to heterogeneity cannot be determined by the population-level experiments performed.

The morphology of the cells in retentostat cultivation is clearly heterogeneous. The observed chain formation in **Chapter 4** is most likely caused by suppression of

SigD-regulated genes coding for autolysins (**Chapter 5**), of which the expression is heterogeneous (Cozy and Kearns, 2010). The benefits of the chain formation under (near-)zero growth conditions are not clear. In respect to the encountered caloric restriction it seems sensible to reduce energy expenditures, as is reflected in general in the expression levels of genes involved with biosynthesis (**Chapter 5**). The energy that is not spent on production of enzymes and hydrolysis of the cell wall for separation of cells, can now be used for maintenance of cellular functions essential to remain viable. On the other hand, appearance of motile cells to migrate towards more nutrient-rich environments can also provide means for survival, as occurs during the transition to stationary phase (Mirel et al., 2000; Rao et al., 2008). There is a possibility that a minority of the cells in the retentostat is motile, as microscopic images show that some cells are not residing in chains (**Chapter 4**). This is in coherence with the notion that *B. subtilis* cultures are found to be differentiated into two subpopulations with cells either ON or OFF for σD-dependent gene expression (Kearns and Losick, 2005; Chai *et al.*, 2010). Such a small proportion of cells expressing motility-genes will not be detected using a population-wide transcriptome analysis. The use of green fluorescent protein (GFP) fused to a promoter of interest has been proven to be a valuable tool in single cell analysis (Chalfie et al., 1994; de Jong et al., 2012; Smits et al., 2005; Southward and Surette, 2002; Valdivia and Cormack, 2005; Veening et al., 2008b) and single-cell studies in the context of retentostat cultivation will yield more detailed information regarding expression of specific genes in sub-populations. The transcriptome analysis in this thesis provides a valuable overview of gene regulation occurring at (near-)zero growth conditions and brings entry points for future detailed studies.

Under diauxic growth conditions, where growth limitation by glucose exhaustion is solved by utilization of available secondary carbon sources, the expression of these genes can be heterogeneous (**Chapter 6**; (Kotte et al., 2014; Solopova et al., 2014). Therefore it might very well be that the observed regulation on transport and utilization of carbohydrates and amino acids is heterogeneous under (near-)zero growth conditions, too.

Retentostat cultivation of *B. subtilis*

A bioreactor consists of many parts, and with a typical experimental duration of more than 40 days, there are many risks of failure that render a retentostat cultivation useless for further analysis. A number of adaptations to the retentostat setup, such as the installment of a dropper unit to prevent *B. subtilis* cells from growing in the medium feed-line, were required for prolonged retentostat cultivation (**Chapter 4**). Next to solutions involving technical changes to the bioreactor, additions to the experimental setup could also help to optimize the retentostat cultivation process. The risk of contamination of the culture with another microorganism increases with the prolonged duration of the retentostat cultivation. The use of GFP enabled us to visually identify *B. subtilis* cells from unwanted contamination in the retentostat. The cultured *B. subtilis* strain contained a fusion of the constitutive ribosomal gene promoter P*rrnB* with GFP, yielding cells that were fluorescent at any time. The GFP variant used was GFP+, an improved version of the regularly used GFPmut1. **Chapter 3** describes a benchmark of seven GFP variants in *B. subtilis* and two additional model-organisms *L. lactis* and *Streptococcus pneumoniae*. The outcome that variants exhibiting the highest fluorescence signal in *B. subtilis* were actually codon-optimized for *S. pneumoniae* and *vice versa*, shows there is still room for improvement in the area of codon optimization. Together with the great variance in phenotypic noise strength of each GFP, this underlines that the GFP variant needs to be carefully chosen per particular experiment. A GFP with low phenotypic noise strength would be more suitable for a single-cell experiment investigating heterogeneity. One of the GFPs with a stronger fluorescence signal would be more applicable for visualization of weak promoter activity. As determined by the benchmark, GFP+ is roughly twice as fluorescent as the regularly used GFPmut1 in *B. subtilis*. Certainly in combination with the strong *rrnB* promoter it is useable for positive identification of *B. subtilis* in retentostat cultures.

Retentostat cultivation of spore-forming organisms such as *Aspergillus niger* resulted in spore formation (Jørgensen et al., 2010). To achieve very low growth rates in a retentostat, it is required that no loss of metabolically active biomass occurs (Boender et al., 2009; Jørgensen et al., 2010). The fact that newly formed spores are dormant and are able to leave the bioreactor through the filter, prevents near-zero growth rates from being reached. The sporulation-negative *sigF*-deletion strain

therefore enabled the study of near-zero growth rates of *B. subtilis*, achieved during retentostat cultivation. Under sporulation conditions the genotype and phenotype of the *sigF* mutant has been characterized (Dworkin and Losick, 2005; Fawcett et al., 2000; Steil et al., 2005; Wang et al., 2006). **Chapter 2** confirms that part of the sporulation network in our *B. subtilis* 168 *sigF*$^-$ strain is disabled and that the strain is unable to form spores. To get a view on regulatory networks operating under zero-growth conditions that is as complete as possible, it is important to influence nothing else but part of the sporulation network. Importantly, **Chapter 2** shows that the disruption of *sigF* has only a minor effect on gene expression during vegetative growth, when sporulation is not initiated in the great majority of the cells. Therefore, the *sigF* mutant strain is considered suitable to study the adaptation of *B. subtilis* to near-zero growth conditions. Although the use of the *sigF* strain 'hides' the part of the sporulation network that is direct or indirect dependent on presence of SigF from sight, and this might be seen as an unnatural situation, it is still possible to monitor early sporulation genes. The ability to still monitor whether cells enter the non-reversible pathway of sporulation, yields a situation as close as possible to the wild type. Under circumstances where spore-formation would make it impossible reach the zero-growth condition that is to be studied, the partial disabling of the sporulation network seems like a fair compromise. Whether this decision is truly a compromise, or rather -as we expect- a pure necessity, needs to be determined by performing a retentostat study on a *B. subtilis* wild-type strain.

Industrial use of zero-growth *B. subtilis*

Microorganisms have many applications as cell factories for the production of chemicals. *B. subtilis* and its close relatives are used e.g. for production of vitamins, enzymes, antibiotics, pharmaceuticals (Baek et al., 2012; van Dijck, 1998; Olmos-Soto and Contreras-Flores, 2003; Papagianni, 2012; Westers et al., 2004; Zamboni et al., 2003). Traditionally, various microbes are applied in food production such as wine fermentation by *Saccharomyces cerevisiae* (Mauricio et al., 2001), cheese ripening by lactic acid bacteria (Smit et al., 2005), and natto fermentation by *B. subtilis* (Steinkraus, 2004). Under these industrial conditions, the nutrient availability is limited and consequently, growth rates are very low. The knowledge on zero-growth cultures therefore could be of potential use for optimization of these industrial

processes. Additionally, knowledge on zero-growth is valuable in the application of microorganisms as cell factories, since they are self-replicating catalyzers and biomass can be a non-desirable byproduct (Papagianni, 2012; van Verseveld et al., 1986). The uncoupling of growth from metabolic product formation, using zero-growth cultures that maintain productivity, would then be beneficial for the product yield. Near-theoretical yields were achieved in anaerobic retentostat cultures of *S. cerevisiae* (Boender et al., 2009) and *L. lactis* (Ercan et al., 2013), where virtually all energy substrate was converted to the main fermentation products. For example, yields of the major byproducts of bioethanol production by *S. cerevisiae*, yeast biomass and glycerol (Brandberg et al., 2007), were negligible in these retentostat cultures (Boender et al., 2009). This illustrates the benefit of zero-growth cultivation for catabolic product yield improvement. However, if future gas-exchange analysis on aerobic *B. subtilis* retentostat cultures confirms that all energy substrate was indeed converted to carbon dioxide and water (**Chapter 4**), the application of *B. subtilis* for catabolic product formation in aerobic carbon-limited retentostat cultures is questionable. Regarding catabolic production value, retentostat cultivation could possibly be better used under anaerobic and/or energy excess conditions when metabolites such as lactate, acetate, acetoin, ethanol, succinate and 2,3-butanediol are being formed (Cruz Ramos et al., 2000; Dauner et al., 2001; Nakano et al., 1997).

The observed down-regulation of protein synthesis under (near-)zero growth conditions provides a challenge for synthesis of anabolic products in *B. subtilis,* such as enzymes. Constrain of growth by limitation of a non-energy substrate, rather than energy source supply limitation, could be more promising. This is, however, with the prerequisite that reduced efficiency of energy source utilization and overflow metabolism under these energy excess conditions (Dauner et al., 2001) can be prevented. The increased expression that is observed under retentostat conditions for some genes encoding industrial-relevant enzymes like alkaline-protease and mannose-6-phosphate isomerase could be useful. For their production, retentostat cultivation may prove to be beneficial if the production levels are high enough.

Conclusions and future research

During this PhD project much time and effort was spent on the setup and validation of the retentostat system. The set-up and operation of prolonged retentostat cultures is labor-intensive, however, this cultivation method enables the study of an important domain of microbial physiology that is not experimentally accessible via other methods. The retentostat system used in this thesis is well suited for controlled and reproducible cultivation of *B. subtilis* under extreme caloric restriction. Possibly, it may be applied in more studies on non-growing metabolically active *B. subtilis* cells, since the approach holds value not only for understanding microbial lifestyles in nature, but also for uncoupling growth and product formation in industrial microbes.

To get a better understanding of *B. subtilis* under a condition similar to its natural habitat, and that is relevant for industry, it was investigated how *B. subtilis* adapts to near-zero growth rates imposed by extreme caloric restriction. The power of combining controlled cultivation in retentostats with genome-scale analytical techniques is highlighted by this project. Physiological data confirmed that in a *B. subtilis* retentostat culture virtually all substrate was consumed for maintenance purposes and that the maintenance coefficient is growth rate independent (**Chapter 4**). The reduced building block requirement in adaptation to the reduced substrate availability and decreased growth rate was clearly reflected in the transcriptome (**Chapter 5**). Transcriptional regulation occurred on many cellular processes, mainly transport and utilization of carbohydrates and amino acids, and biosynthesis.

This study would benefit from future integration with metabolome analysis of the culture, as this would give more insight in the metabolic adaptations to (near-)zero growth rates. Although no organic acids were detected in the culture medium by HPLC analysis, the proposed dissimilation of glucose to carbon dioxide and water needs to be confirmed with gas exchange analysis in order to get a complete picture of the carbon balance.

Next to data on the population level, a lot can be learned from single cell experiments. Genes known to be heterogeneously expressed in batch cultivation provide interesting targets for single cell studies using promoter-GFP fusions in retentostat cultivation. Additionally, the regulatory networks involved in adaptation to (near-)zero growth rates, revealed by transcriptomics (**Chapter 5**), can be

screened for heterogeneity using promoter-GFP fusions. Heterogeneity can play an important role in determining protein production yield per cell (Veening et al., 2008b), and screening of genes involved in production of industrially relevant compounds might provide entries for process optimization. Separation of various sub-populations (by e.g. Fluorescence Assisted Cell Sorting (FACS)) may provide even more information as subsequent analysis by transcriptomics will reveal all members of the heterogeneously expressed regulons.

Escherichia coli, *Caulobacter crescentus* and *B. subtilis* suffer from replicative aging, as (a)symmetrically divided cells that possess the older poles exhibit a decreased growth rate, produce less offspring, and are more probable to die (Ackermann et al., 2003; Stewart et al., 2005; Veening et al., 2008a). Since the cell cycle of the cells has tremendously slowed down in long-term retentostat cultivation, the chronological age of individual cells has increased. The retentostat may therefore offer an interesting culturing method for studying aging.

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